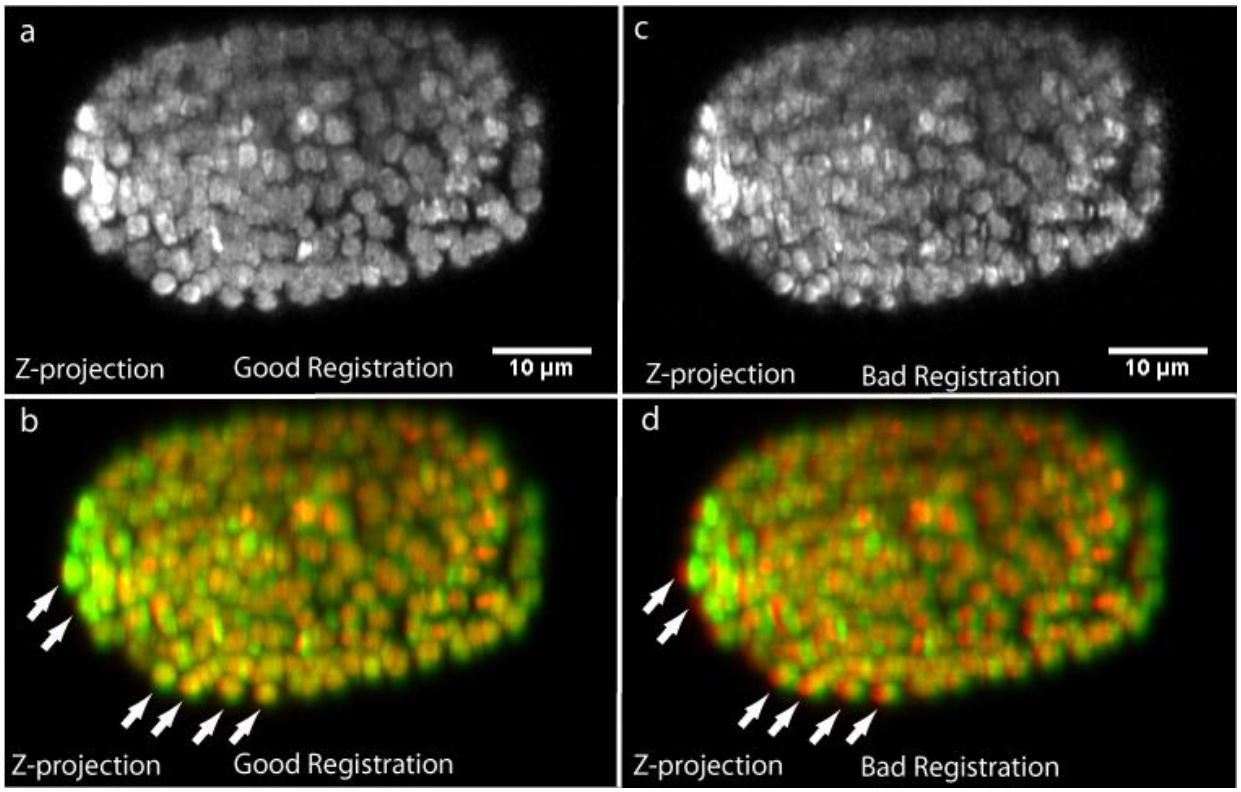
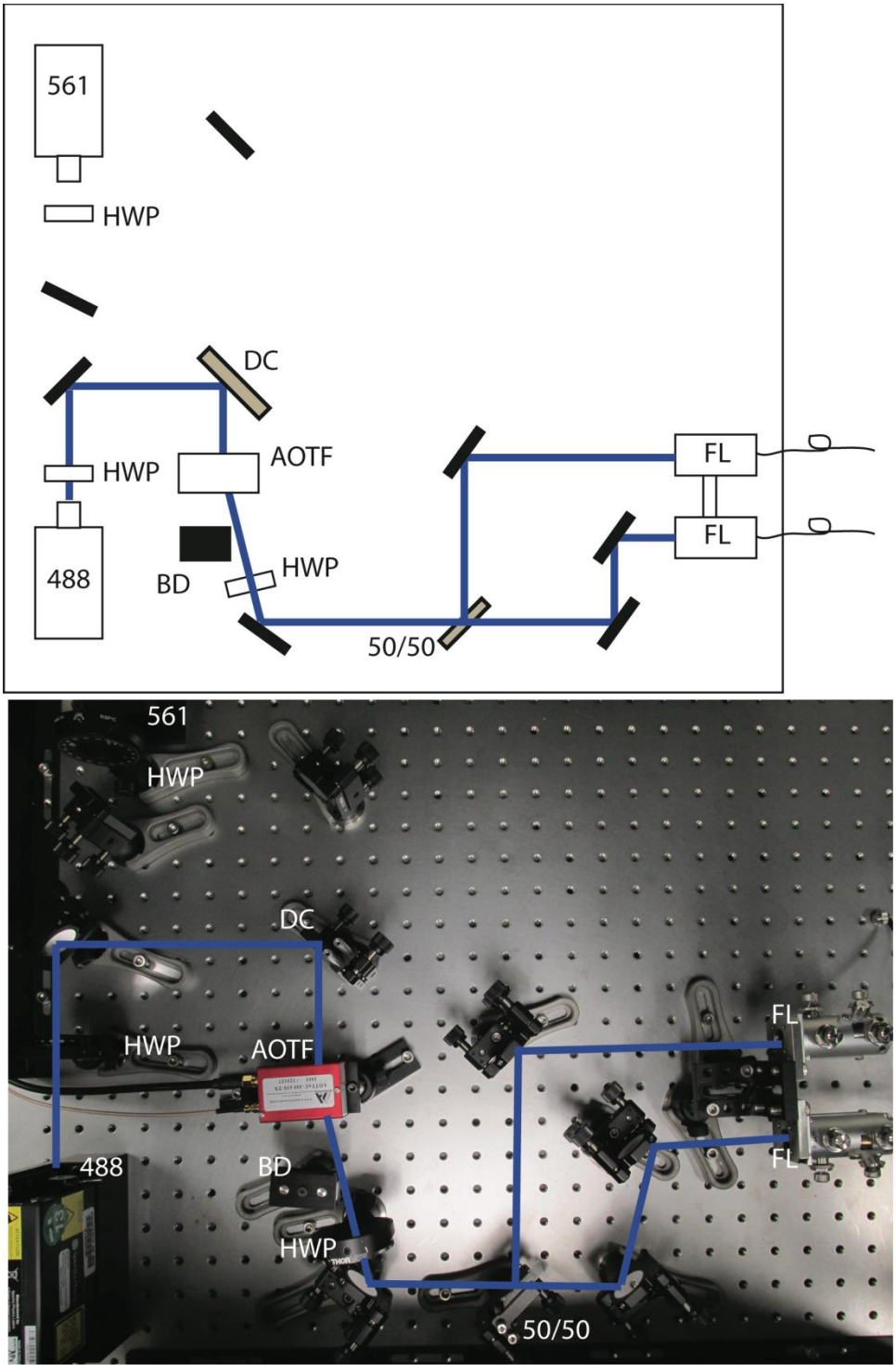
Supplementary Note 1 SF1, Successful and Failed Registrations.



a. Deconvolved image of a BV24 embryo after generation of a registration matrix with a high degree of overlap between the two component views. **b.** Overlap between the two individual SPIM views used to generate the deconvolved image shown in panel **a. c.** Deconvolved image of a BV24 embryo after generation of a registration matrix with a low degree of overlap between the two component views. Note the poor quality of the resulting reconstruction. **d.** Overlap between the two individual SPIM views used to generate the deconvolved image shown in panel **c**. The red channel in **b, d** represents the image from arm A (base image), while the green channel represents the image from arm B (transformed image). Arrows point to cells showing a high (in **b**) or low (in **d**) degree of overlap (visible as an offset in the location of the same cell in the red and green channels, see also **Supplementary Data 1**).

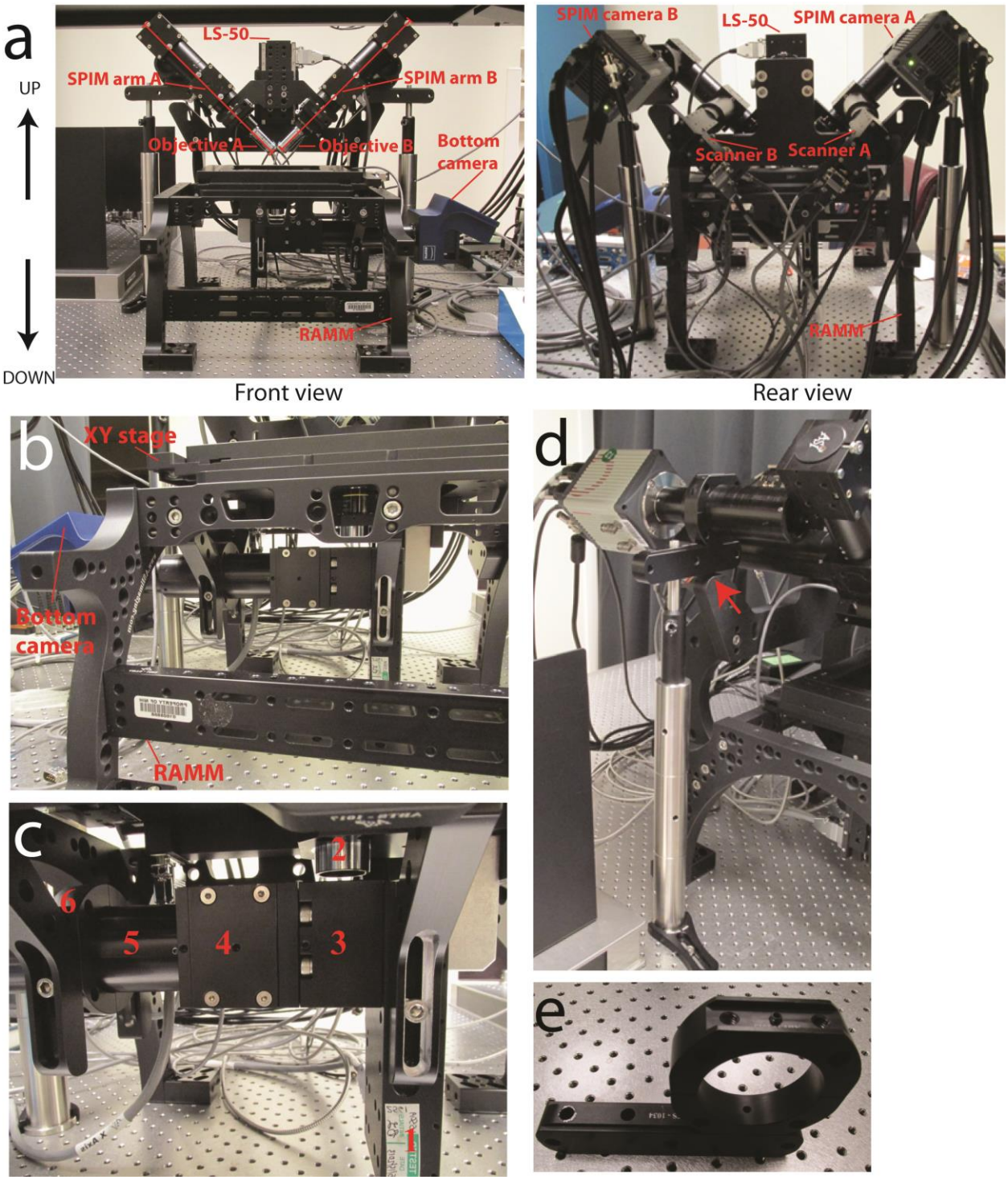
Supplementary Note 1 SF2, Excitation laser launch for diSPIM



Top: schematic showing key optomechanical components. Note that drawing is not to scale. Bottom: Photograph of setup used in protocol. For clarity, only the beam corresponding to the 488 nm laser is shown. All optomechanics are bolted to a 2.5' x 2.5' optical sled (Thorlabs, Cat.

B3030F). 488 nm (Newport, Cyan scientific laser, 100 mW) and 561 nm (Crystalaser, 150 mW) lasers are passed through half-wave plates (Thorlabs, Cat. # WPH05M-488 and Cat. # WPH05M-561), and combined with a dichroic mirror (Semrock, Cat. # Di02-R488-25X36), before transmission through an AOTF (AA Optoelectronics, Cat. # AOTFnc 400.650TN). The half wave plates are mounted in rotation mounts (Thorlabs, Cat. # RSP1C) and are rotated to maximize transmission through the AOTF. The output beam is again passed through a broadband half wave plate (Thorlabs, Cat.# AHWP05M-600) and then directed onto a 50/50 beamsplitter plate (Thorlabs, Cat.# BSW10R). The preceding half wave plate ensures that the beam is evenly split by the beamsplitter. Each output beam is then directed onto optical fiber launches. The fibers provide excitation to each arm of the diSPIM. Abbreviations used in figure: 488: 488 nm laser; 561: 561 nm laser; HWP: half-wave plate; DC: dichromatic mirror; AOTF: acousto-optic tunable filter; BD: beam dump; 50/50: 50% reflecting and 50% transmitting beamsplitter; FL: fiber launch (kineFLEX-Fiber Delivery System, QIOPTIQ).

Supplementary Note 1 SF3, Photographs of major components in diSPIM



a. Photographs of the diSPIM as viewed from the front and back, with major assemblies marked in red. 'Up' (away from coverslip, towards ceiling) and 'down' (towards coverslip, and optical table) directions are defined, and 'A' and 'B' arms are specified. See also **Fig. 1**. **b.** Lower imaging path, emphasizing position relative to the bottom camera, RAMM frame, and XY

sample stage. **c.** Higher magnification view of lower imaging path, with (1) LS-50 translation stage; (2) lower objective holder; (3) lower reflective mirror cube; (4) lower dichroic mirror cube; (5) tube lens; and (6) tube lens bracket marked. **d.** SPIM camera and assembled mount. Tube lens and mirror cube are also visible. **e.** Higher magnification view of tube lens bracket and holder (ASI part # B1034, marked in **d.** with red arrow).

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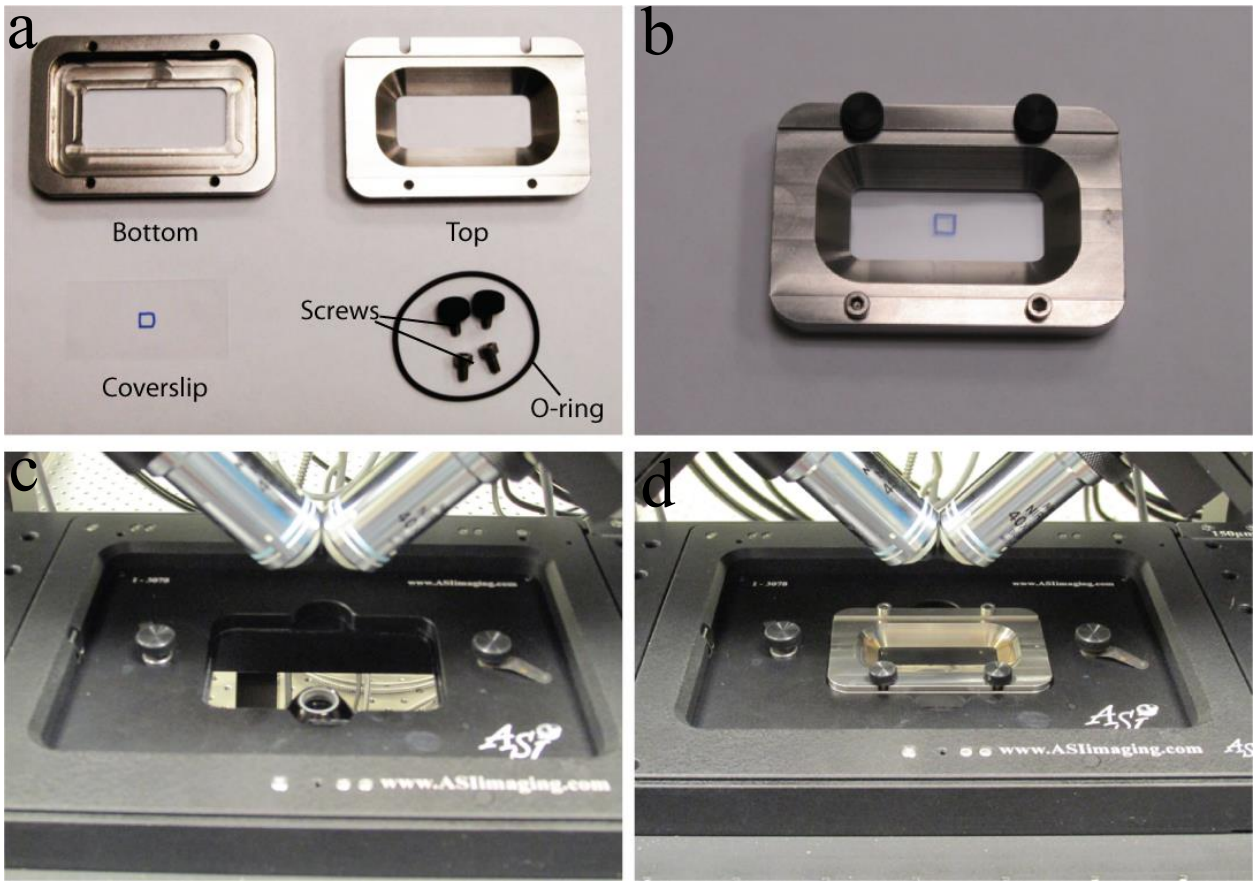
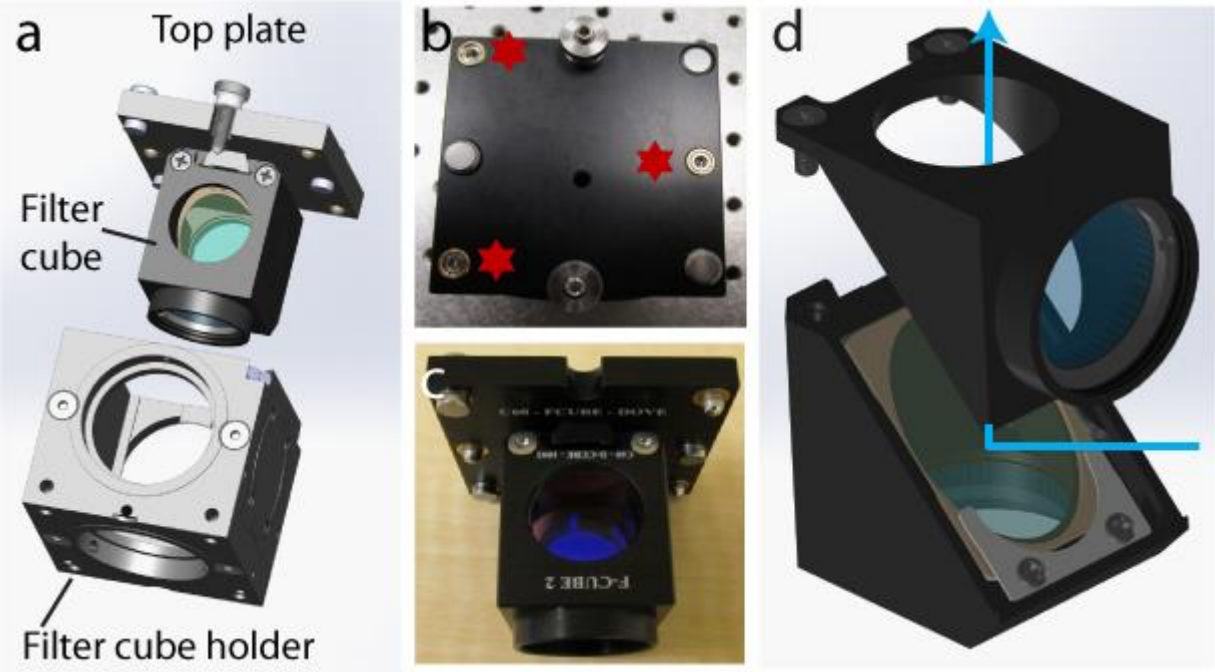
Supplementary Note 1 SF4, **The diSPIM sample chamber**



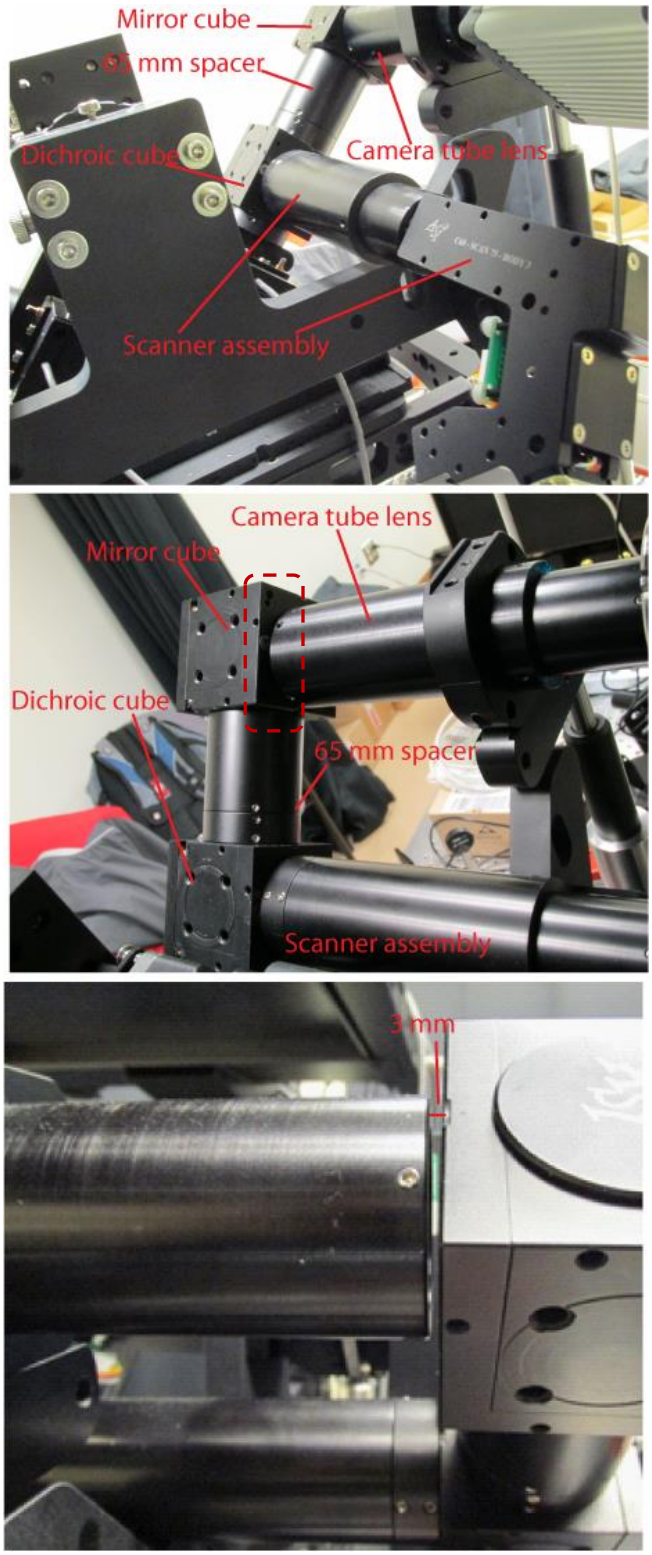
a. The two halves of the disassembled chamber, rectangular coverslip (note marked 4 mm x 4 mm square region in the center), O-ring, and screws. **b.** Assembled sample chamber. **c**. Chamber insert placed in XY stage (without chamber). Objectives are indicated above the insert. **d**. the assembled chamber placed in the chamber insert.

Supplementary Note 1 SF5, diSPIM Dichroic Filter Cube Assembly



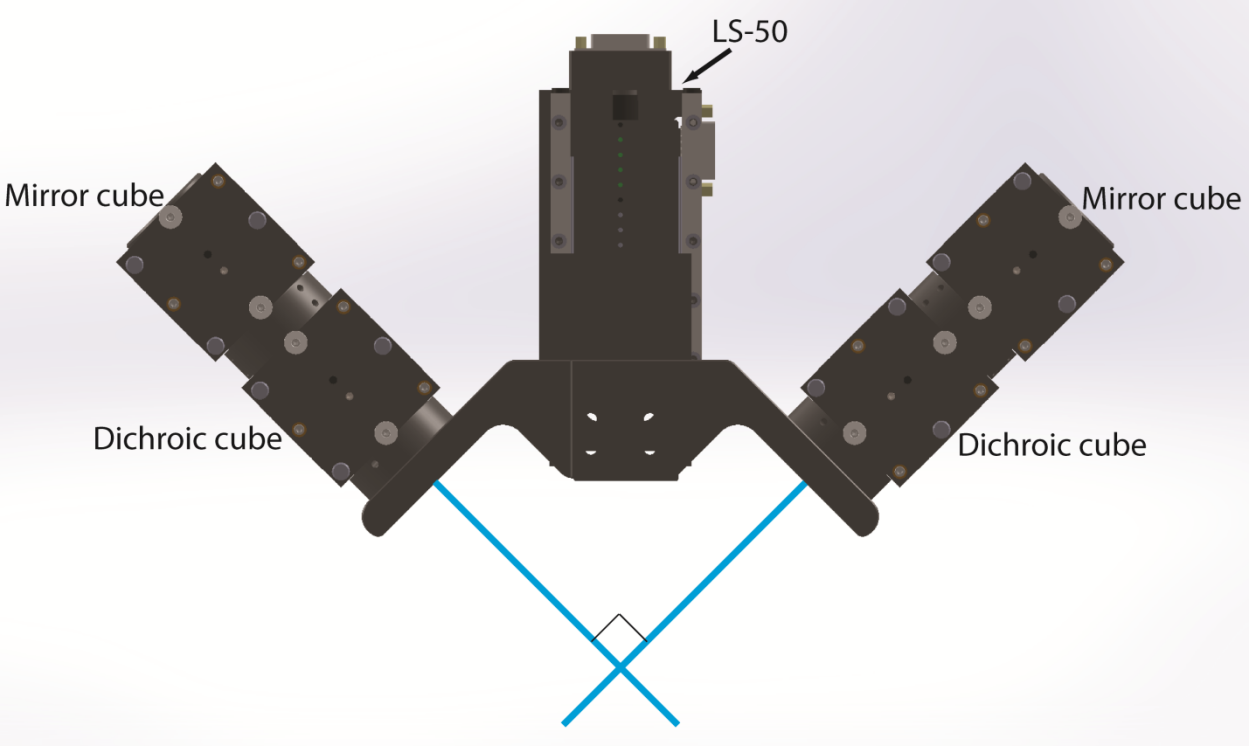
a. Main components in each dichroic filter cube assembly. **b.** Photograph of top plate. Red stars indicate fine alignment screws that, when tweaked, tilt the dichroic filter cube relative to the holder. **c**. Photograph of top plate with dichroic filter cube attached. **d.** Schematic emphasizing filter cube with dichroic. Blue arrow indicates the direction of excitation laser light.

Supplementary Note 1 SF6, Connections between excitation scanners, filter cubes, and cameras



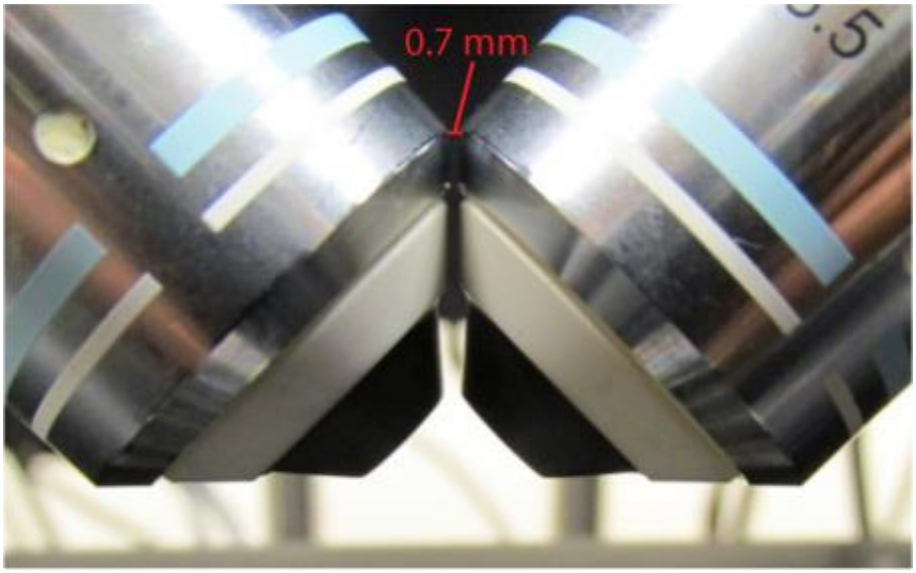
Top and middle: Views of connections between subassemblies in the diSPIM module. The scanner assembly attaches to the dichroic cube holder. The dichroic cube and mirror cube are separated by a 65 mm (50 mm +15 mm) spacer. Bottom: Figure shows the separation between mirror cube holder and camera tube lens (dotted red area in middle panel). Refer to main text for more detail.

Supplementary Note 1 SF7, Intersection of excitation beams from each arm with objectives out



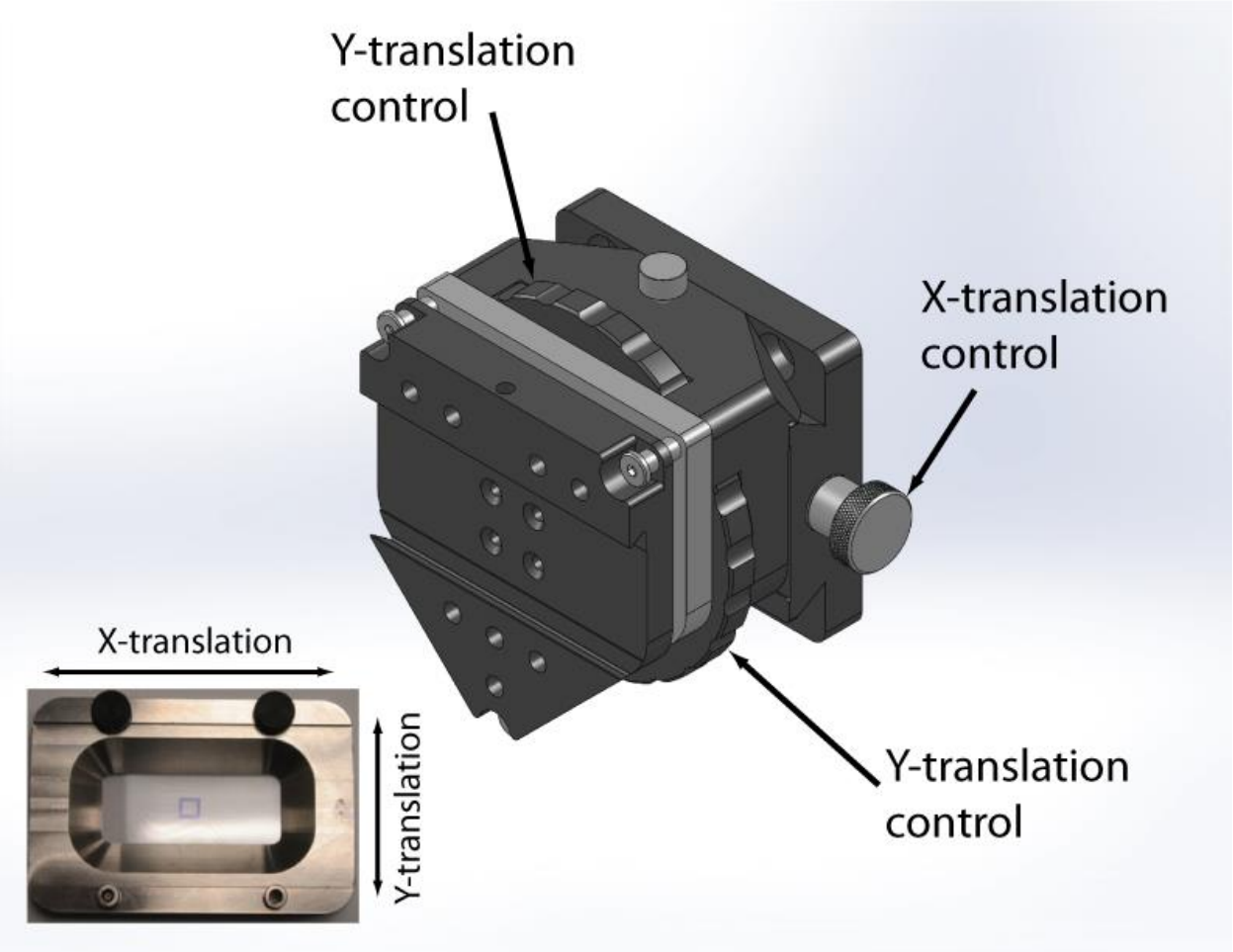
Ideal beam alignment prior to addition of objectives: excitation beams emerge from the center of each aperture on the diSPIM module, with a 90 degree angle between beams at intersection point.

Supplementary Note 1 SF8, Spacing between objectives when aligned



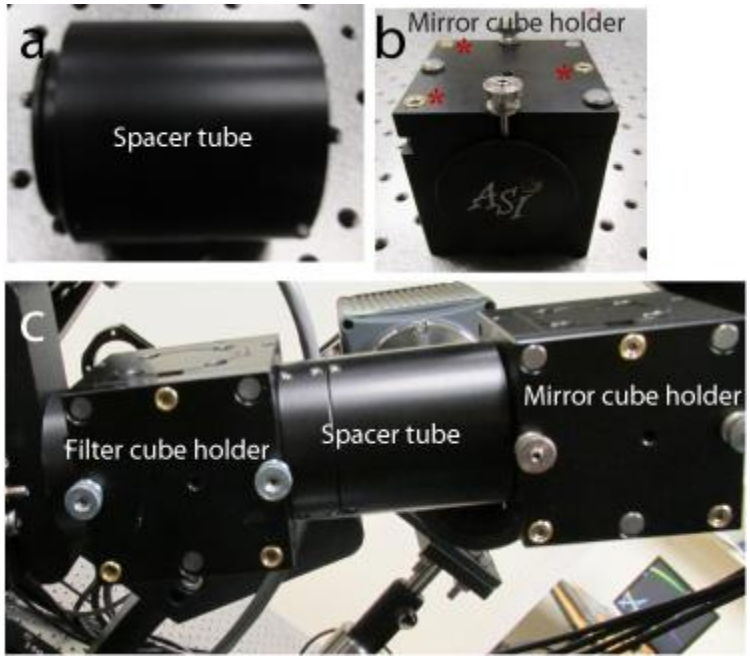
Photograph of correctly aligned objectives. At the indicated position, objectives are ~0.7 mm apart.

Supplementary Note 1 SF9, CDZ-1000 translation stage for coarsely adjusting lateral position of SPIM beams relative to coverslip



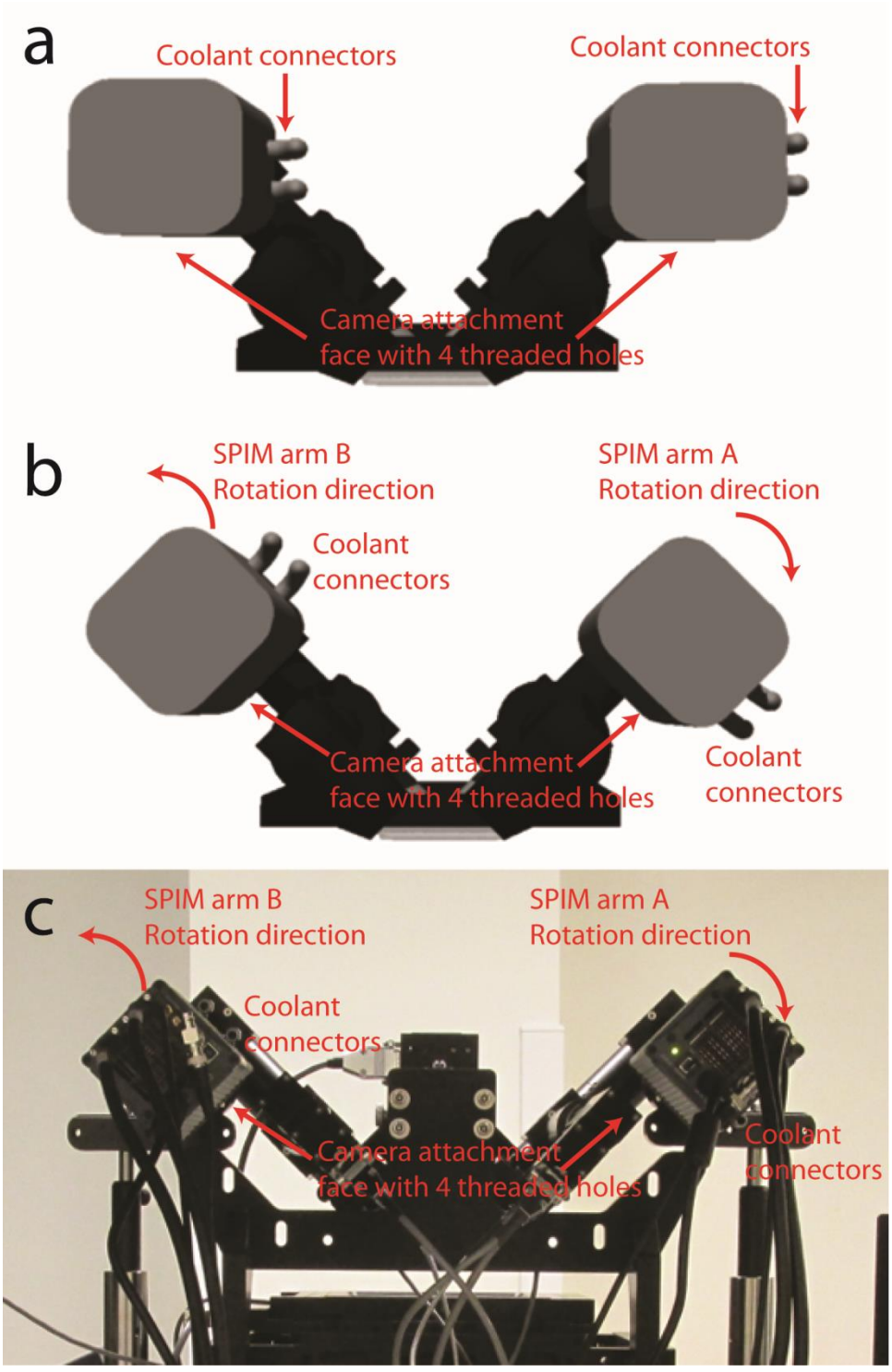
Schematic of the CDZ-1000 translation stage, highlighting adjustments that laterally change the position of the diSPIM module above the coverslip. Inset: translation directions relative to coverslip geometry.

Supplementary Note 1 SF10, SPIM mirror cube assembly



a. 50 mm spacer tube used to separate mirror cube holder from filter cube holder. **b.** Mirror cube holder containing mirror. Red asterisks indicate alignment screws that are used to tilt mirror cube relative to holder. **c.** Filter cube holder, spacer tubes (50 mm + 15 mm), and mirror cube holder.

Supplementary Note 1 SF11, Orienting the diSPIM cameras



a. Orientation of both cameras with base plates directed towards the coverslip. **b.** Cameras after rotation. **c.** Photograph to accompany **b**, showing camera position after rotation. In all three panels, the diSPIM is viewed from behind.

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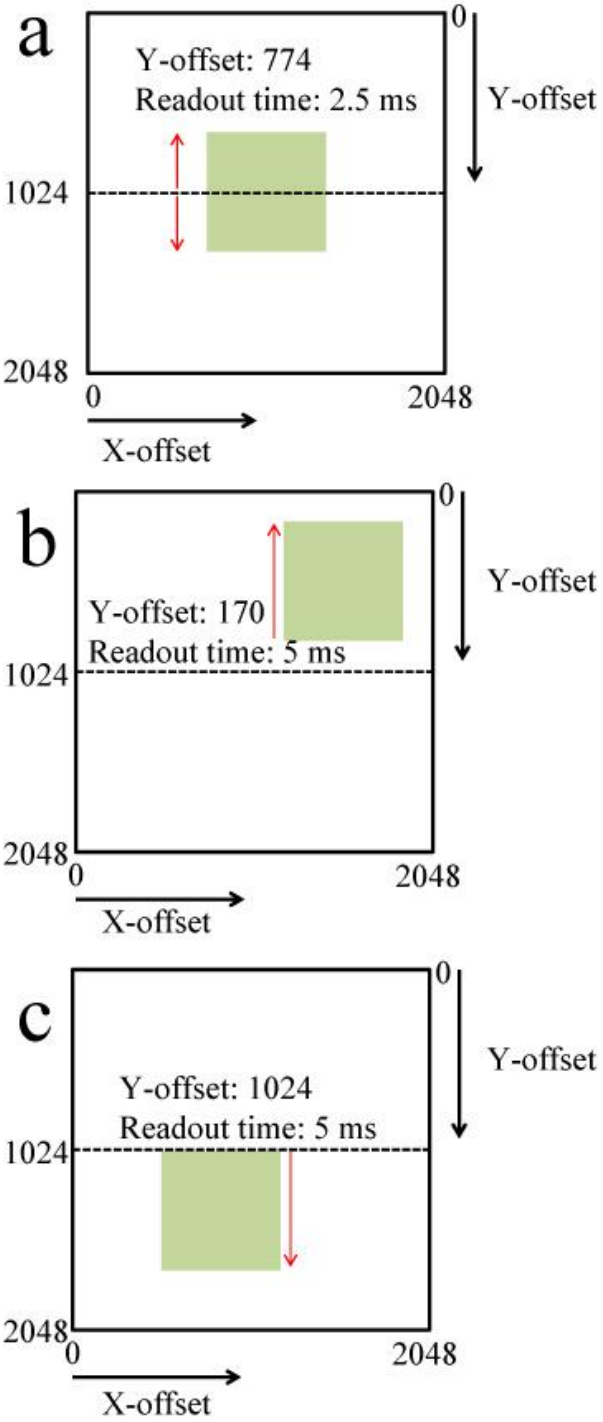
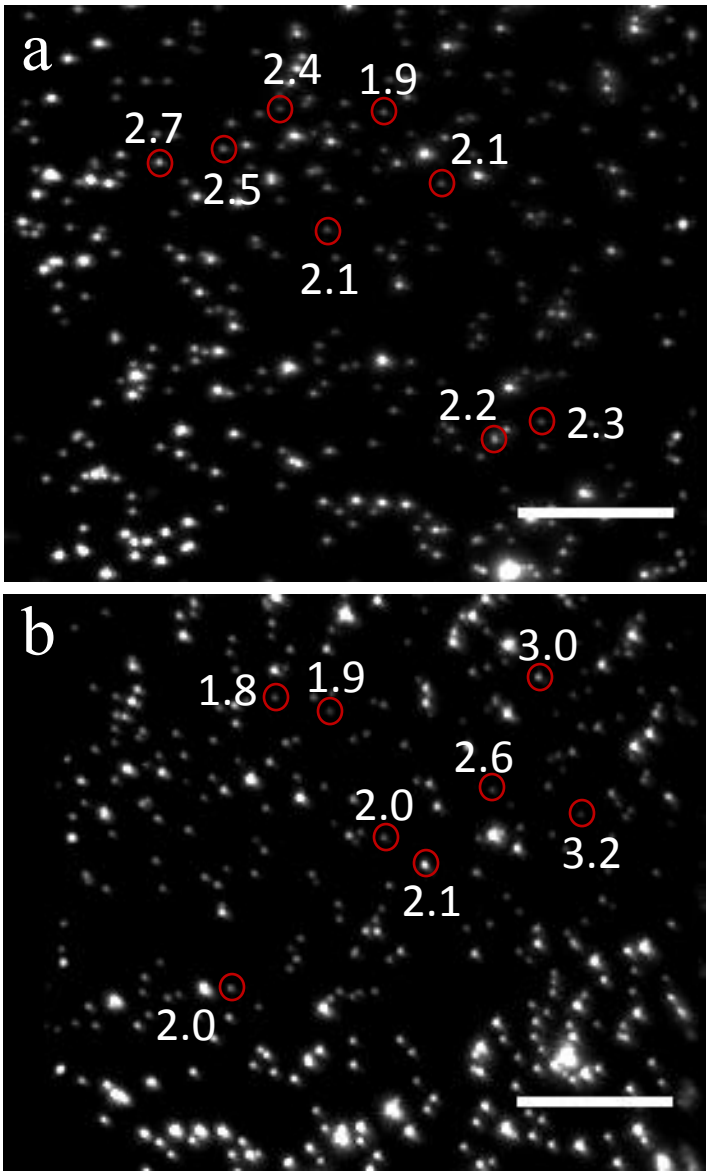
Supplementary Note 1 SF12, ROI Selection in SPIM Cameras



a. The Hamamatsu Flash 4.0 cameras used for SPIM detection employ a 'rolling shutter' readout mode. Positioning the field of view (here a 500 x 500 pixel subarray, green box) in the center of the chip improves readout speed, as each half of the chip is read out independently (red arrows). Coordinate axes and offsets referred to in the main text are also indicated. Panels **b** and **c** indicate

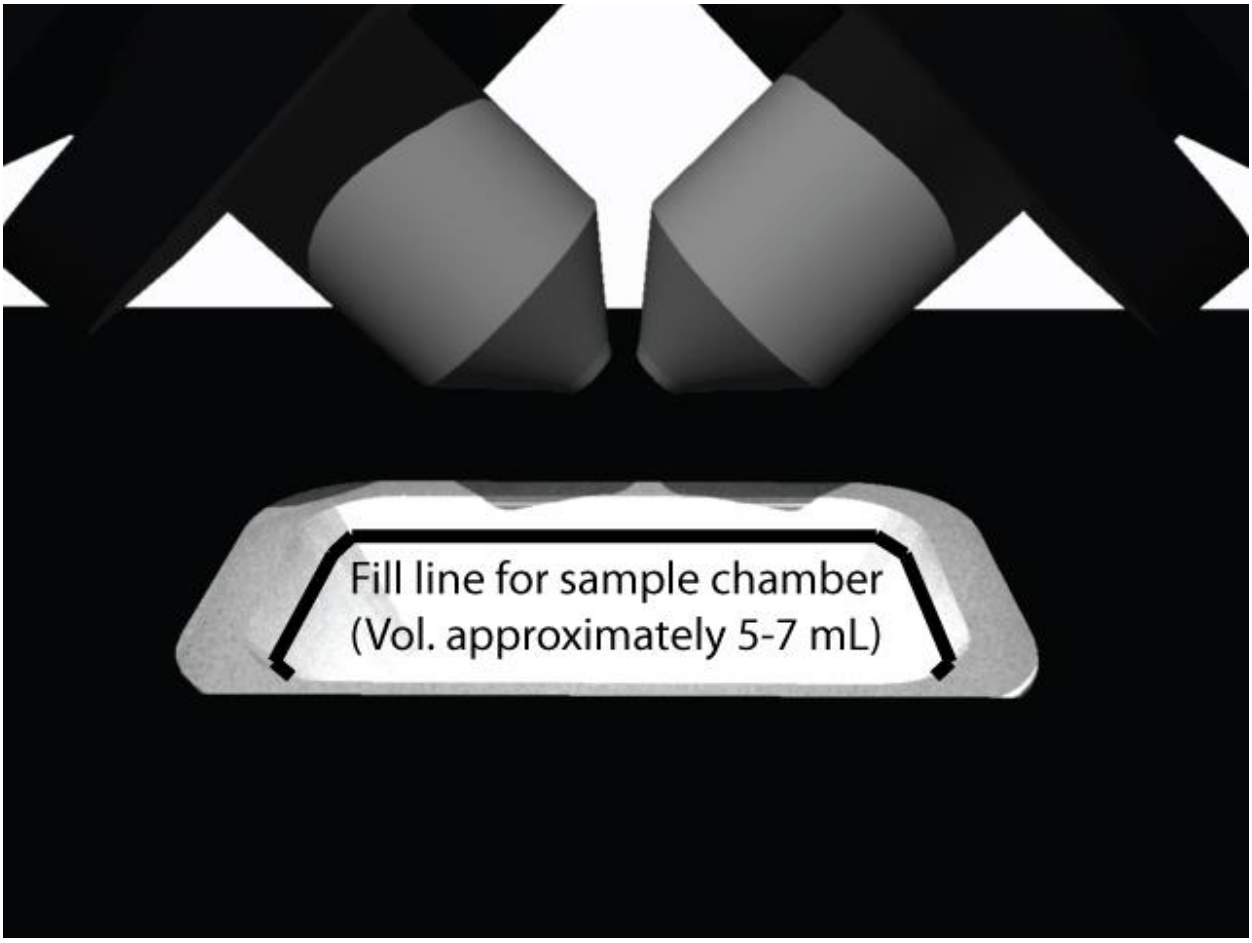
suboptimal placement of the ROI, as the entire subarray must be read out along one half of the chip, reducing speed 2x. The relevant Y-offsets for these cases are also indicated. Note that readout speed does not depend on the X-offset or number of pixels in the X direction.

Supplementary Note 1 SF13, Light sheet thickness measurement across the field of view for both views



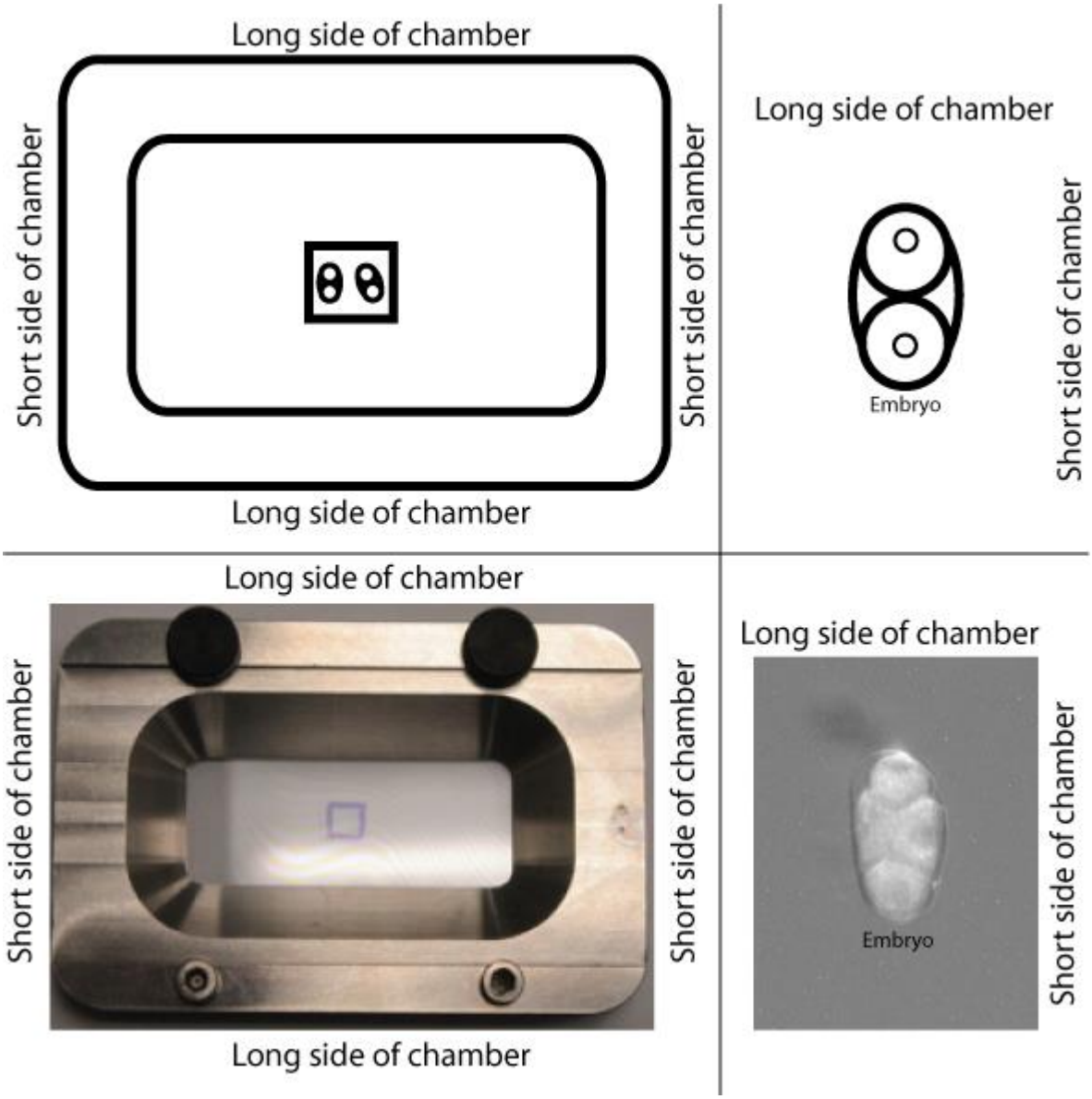
Maximum intensity projections of an imaging volume taken of a fluorescent bead layer, as viewed through arm A (**a**) and arm B (**b**). The light sheet thickness (in μm) measured at different beads, at different locations in the field of view has been marked in each view. Scalebars: 10 μm.

Supplementary Note 1 SF14, Fill line for sample chamber



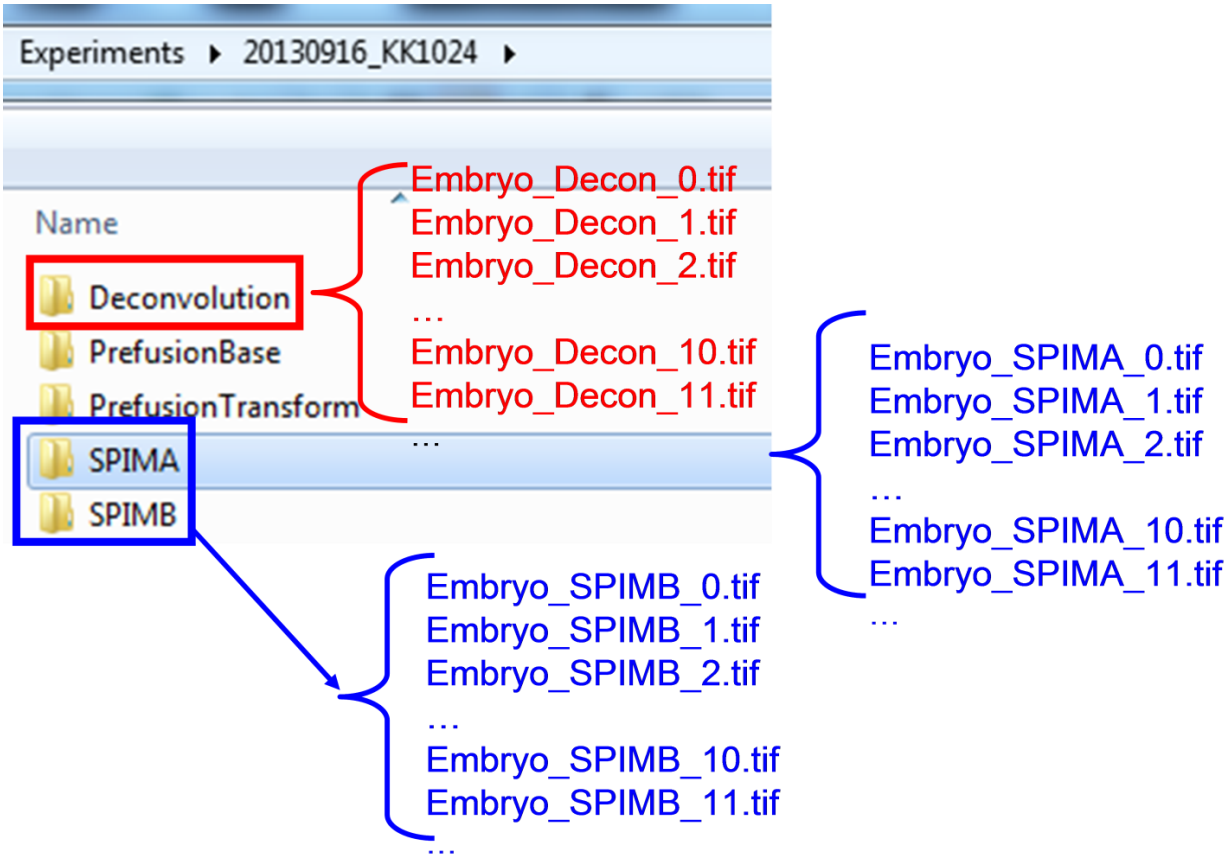
A schematic showing how much of the imaging sample chamber should be filled when mounting a sample. Underfilling decreases sample viability and worsens image quality, while overfilling can cause spills.

Supplementary Note 1 SF15, Preferred embryo orientation for diSPIM



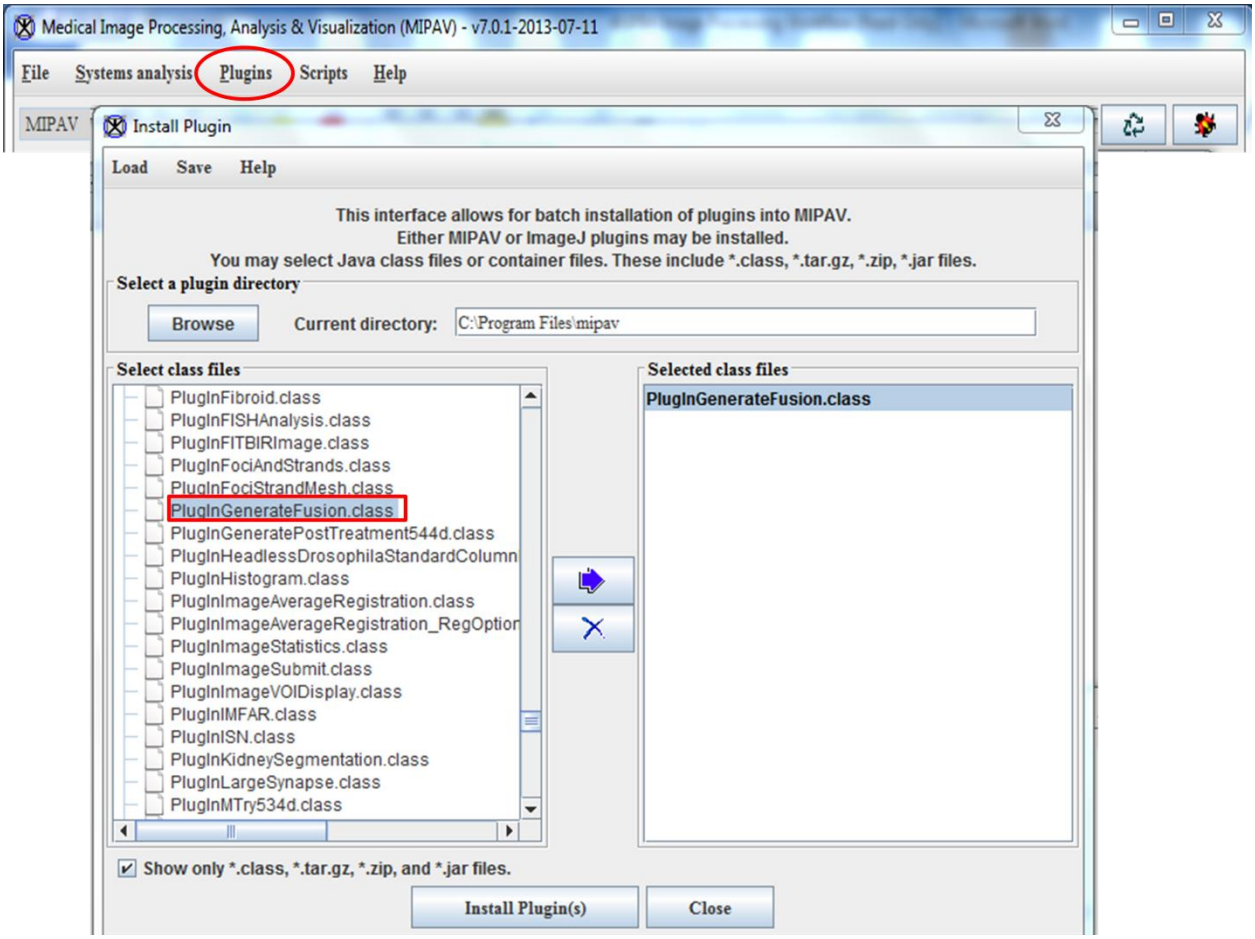
Schematics (top row) and photographs (bottom row), indicating preferred, vertical orientation of *C. elegans* embryo as viewed from above chamber. Note that figures are not to scale.

Supplementary Note 1 SF16, Organization of data for registration/deconvolution.



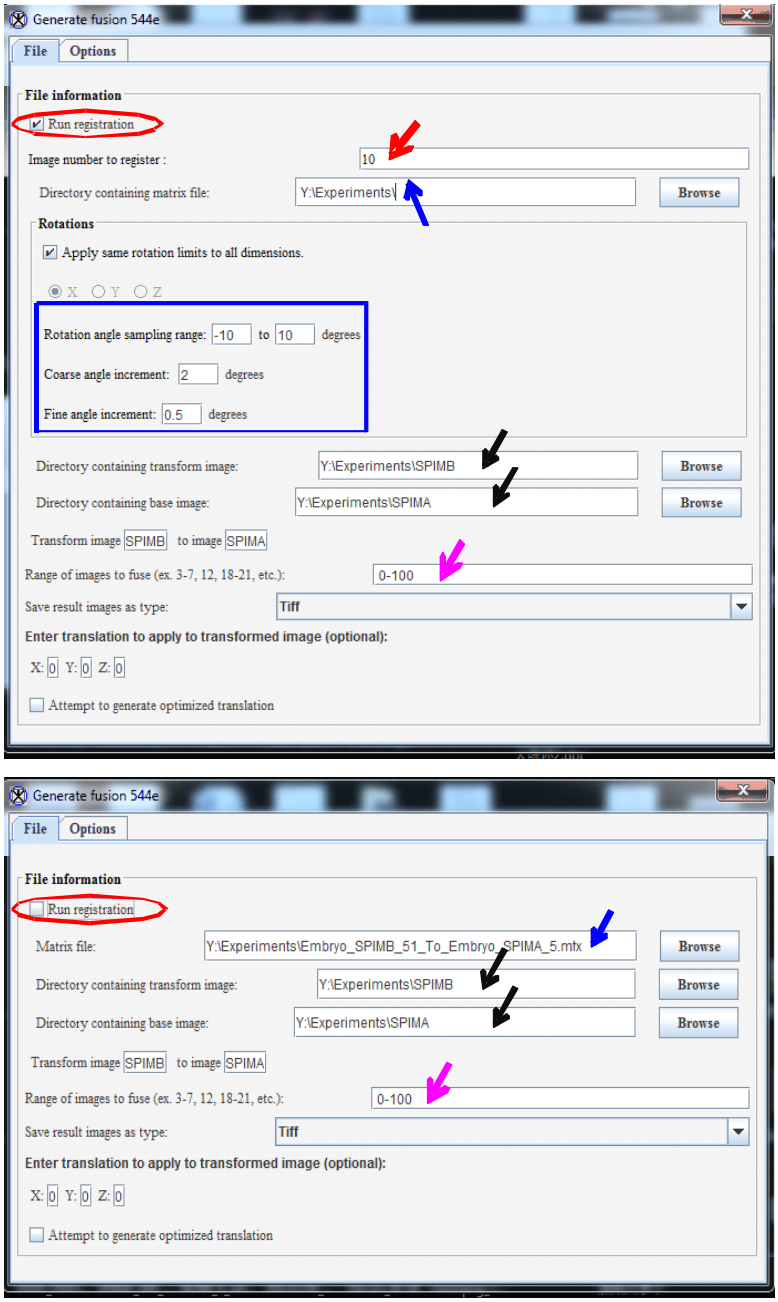
Raw data are organized into two subfolders, corresponding to the two collected views (blue text). An output folder containing the deconvolution results (red text) is also shown.

Supplementary Note 1 SF17, Installation of the diSPIM plugin into MIPAV.



See step **101** in main text for accompanying description.

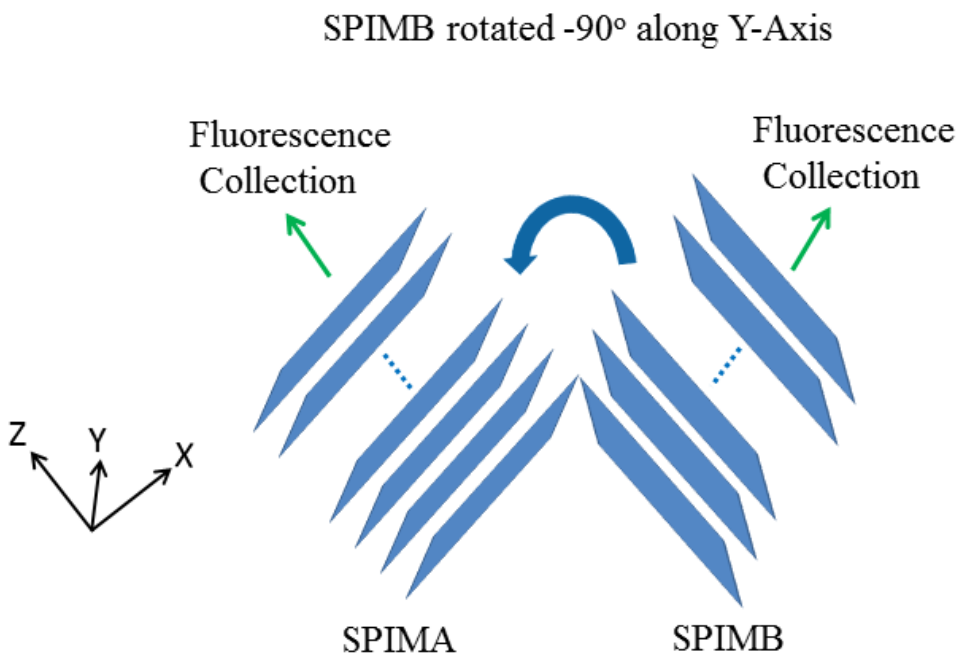
Supplementary Note 1 SF18, Parameter Settings in diSPIM plugin, File tab menu.



Top: Running the plugin to find a transformation matrix, by checking the 'Run registration' box (red oval). Blue arrow: user defined path indicating the directory that the transformation matrix should be saved in. Red arrow: number indicating which volume to use in performing the registration operation. Blue rectangular box: parameters that specify angular range to search over when performing the registration. Bottom: Supplying a user-defined transformation matrix, by leaving the 'Run registration' box unchecked (red oval). Blue arrows: path containing the user-defined registration matrix. Black arrows: user defined path indicating location of data. The 'transformed images' are the datasets that will be rotated into the same coordinate system as the

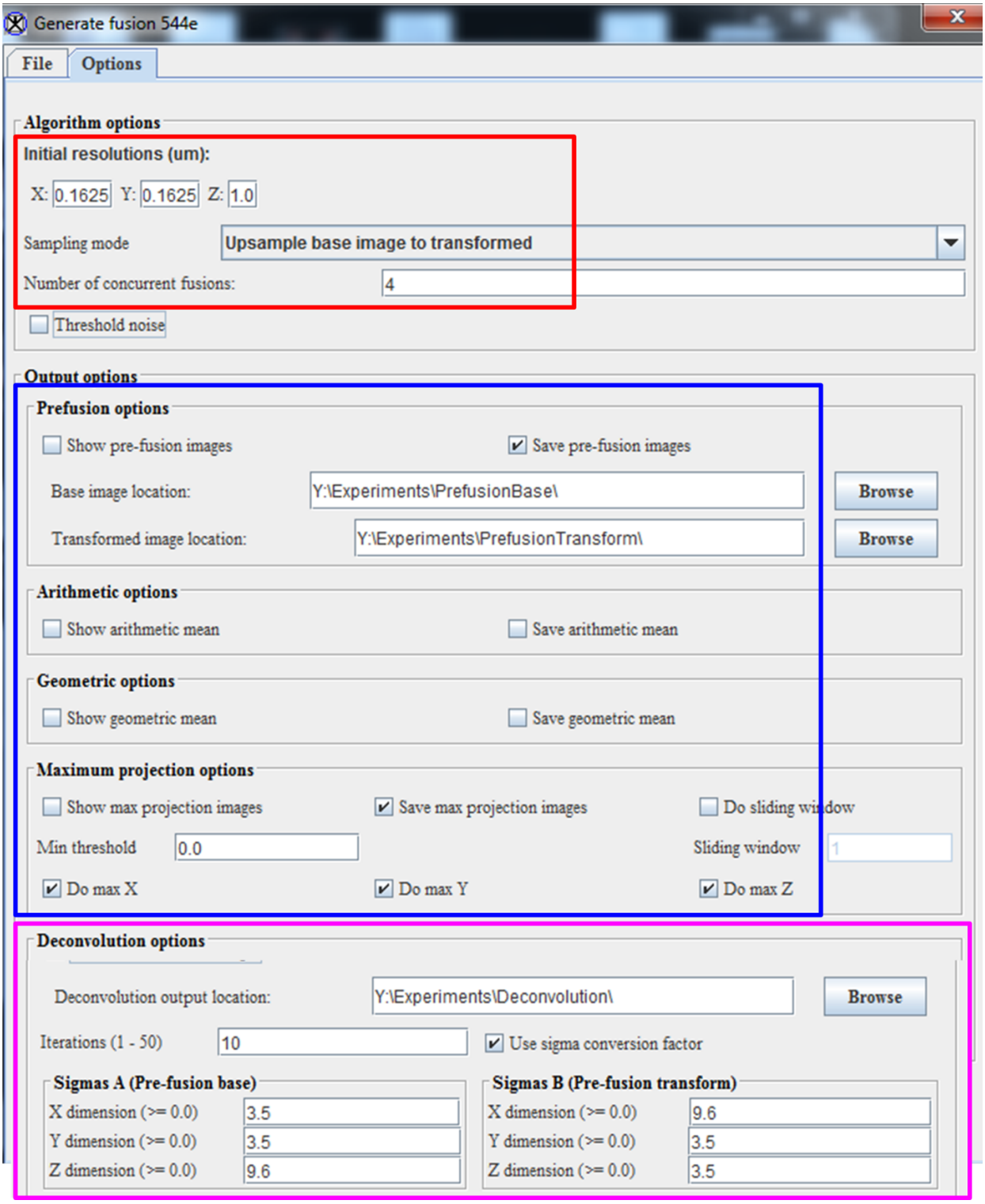
‘base images’. Purple arrows: user defined range, specifying the volumetric timepoints on which to perform the transformation and joint-deconvolution operations.

Supplementary Note 1 SF19, Schematic showing the rotation of SPIM view B into the reference frame of SPIM view A.



Schematic represents both views as acquired by diSPIM. During the image processing steps, the arm B view will be rotated by -90 degrees along the Y-Axis to have the same coordinate system as in arm A. See Step **106** in main text for accompanying details.

Supplementary Note 1 SF20, Parameter Settings in diSPIM plugin, Options tab menu.



Parameters in the 'Algorithm options' box (red rectangle) include the imaging pixel size and the number of concurrent fusion operations (equal to the number of CPU cores the user allocates). In

the ‘output options’ box (blue rectangle), specify a number of quick calculations that can be displayed and saved prior to deconvolution (including maximum intensity projections and arithmetic or geometric means). In the GPU-based ‘Deconvolution options’ box (purple rectangle), set the iteration number and the spatial extents (sigma value) of the assumed Gaussian point spread functions in each view, as well as the output path for the joint deconvolution.

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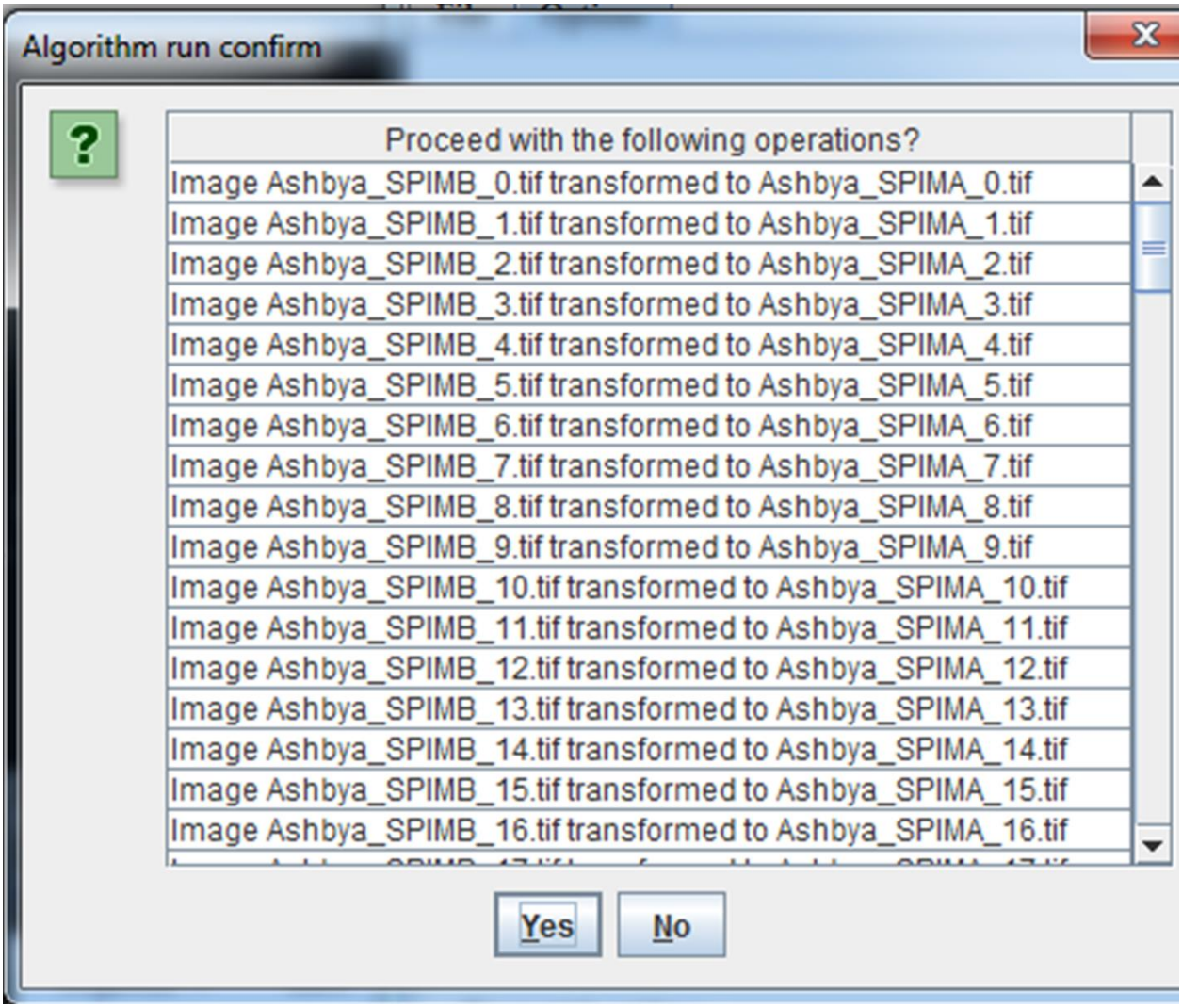
Supplementary Note 1 SF21, Confirmation screen before joint-deconvolution begins.



See accompanying description in step **108** in main text.

Supplementary Table 1, Accessible values of beam waist and field of view using excitation scanner

OBJECTIVE		LASER FOCUS	
40X	10x		
BFP Beam Diameter		Field of view 2 Zr @ λ=500nm	Waist = 0.85 * λ/(2 * NA)
(mm)	(mm)	(µm)	(µm)
3	12	6.3	0.7
2.5	10	9.1	0.9
2	8	14	1.1
1.5	6	25	1.4
1.2	4.8	39	1.8
1	4	57	2.1
0.8	3.2	89	2.7
0.6	2.4	158	3.5
0.4	1.6	355	5.3
0.3	1.2	631	7.1
0.23	0.9	1073	9.2

Parameter values for Nikon 40x (used in this protocol) and 10x objectives (not used), illustrating dependence of field of view (twice the Rayleigh range Zr) and beam waist on beam diameter at the back focal plane (BFP) of the microscope objective. Values highlighted in yellow are accessible in the scanners used in this protocol, by stopping down the iris (**Fig. 1d**). Beam diameters larger than 2.5 mm are clipped to the dimensions of the scan mirror, and beam diameters much smaller than 0.5 mm may entail prohibitive loss of laser power. Note that the diameter of the iris should be set to ~1.4x the desired diameter at the back focal plane, given the 0.73x magnification between iris and BFP in the current scanner design.

Supplementary Table 2, Additional features required in HP Z820 workstation

HP Part #	Feature
LJ452AV	HP Z820 Workstation Base Unit
QG197AV	HP Z820 1125W 90% Efficient Chassis
LJ456AV#ABA	HP Z820 Localization Kit
QG517AV#ABA	MS Windows 7 Professional 64-bit OS US
A2A33AV	Intel® Xeon® E5-2630 2.30 15MB 1333 6C
A2A47AV	Intel® Xeon® E5-2630 6C 2.30 15MB 1333 CPU-2
QG280AV	64GB DDR3-1600 (8x8GB) 2CPU Registered RAM
C2J78AV	NVIDIA graphics processing unit (GPU) card, Quadro K5000 4GB DL-DVI(I)+DL-DVI(D)+DP+DP
QJ688AV	2TB 7200 RPM SATA 1st Hard Drive
QG252AV	HP SATA Blu-ray Writer Optical Drive
QG096AV#ABA	HP USB Standard Keyboard
QG247AV	HP USB Optical Scroll Mouse
A7E47AV	HP Dual Processor Air Cooling Kit
B6S40AV	No Recovery Media Included
QG255AV	HP Single Unit Packaging
D1A00AV	HP DisplayPort To DVI-D Adapter (2-Pack)

Supplementary Table 3, Complete list of ASI hardware needed for the diSPIM

ASI Part #	Description
DiSPIM-K	Contains main mechanical and optical components for the diSPIM. Includes: **Qty. 2 MIM-Tube-K (200):** 200 mm tube lens for SPIM camera. **Qty. 2 MIM-Tube-K (160):** 160 mm tube lens for scanner. **Qty. 2 C60-D_Cube:** Cube for mounting dichroic mirror and emission filter (referred to in the protocol as 'dichroic cube'). **Qty. 2 C60-25mm_CUBE-RA-MIRROR:** Cube for mounting reflective mirror, that reflects emission towards SPIM camera (referred to in the protocol as 'mirror cube'). **Qty. 4 C60-BEAMSPLITTER-II:** Dichroic cube and mirror cube holder with precision alignment adjusters (2 for each diSPIM arm). **Qty. 2 C60-RAO:** Circular mount attached to diSPIM arms for holding C60-Beamsplitter-II. **Qty. 2 RAO-005:** Jam nuts to lock the diSPIM mount. **Qty. 2 C60-EXT-15:** 15 mm extension tube. **Qty 2. C60-EXT-50:** 50 mm extension tube. This (and the 15 mm extension tube) are bolted together for a total extension of 65 mm. These tubes are used to ensure sufficient separation between the cameras and diSPIM module, so that they do not collide as the diSPIM module moves up and down. **Qty. 1 RAO-0004:** Objective holder for diSPIM arm B. **Qty. 1 RAO-0023:** Objective holder for diSPIM arm A. **Qty. 1 PZMAG-AOA:** Objective adjuster for diSPIM arm B. **Qty. 1 RAO-0044R&L:** Two arms attached to LS-50 and holding diSPIM module. **Qty. 1 LS-50M:** Motorized translation stage for vertically translating the diSPIM module. **Qty. 2 MM-SCAN_1M:** Micro-mirror scan head. Two-axis unit with image plane XY scanner mirror, one FC/PC SM laser input. Contains the scanner that creates the light sheet and sweeps it through the sample for volumetric acquisition. Scanner provides focused scan-plane at female C-mount. Uses TGMM4 controller for electronic control of the scanner, and additionally provides manual aperturing of beam at back focal plane with an adjustable iris. Provides a 15 mm diameter scan field at C-mount. >300Hz X and Y bandwidth. **Qty. 1 16 Slot TIGER Controller:** Controller electronics for scanners, piezos, stage, and LS-50 stages. Includes TG16, TGCOM, Qty 2. TGDCM2, SA JOY+ZF, control for DC servo motors, com card w/USB, power supply and joystick with two knobs.
SPIM-MOUNT	Includes **Qty. 2 B1032:** Side bars attached to RAMM for holding B1033. **Qty. 1 B1033:** Crossbar which holds CDZ-1000. **Qty. 4 B1013 :** Tube lens holder for SPIM camera and scanner (2 for each

	diSPIM arm). **Qty. 2 B1034:** Bar for holding B1013.
SPIM_RAMM	Support framework and mounting components for the microscope system Includes: **Qty. 2 ARCH:** Side arch for holding X-Y stage. **Qty. 4 FOOT:** for bolting the RAMM down to the optical table. **Qty. 2 B1017:** Crossbar attached to arch, holds sample stage. **Qty. 1 B1013:** Tube lens holder for bottom imaging path. **Qty. 2 B1016**: "Drop arms" that attach the LS50 and lens tube rings to the RAMM frame. **Qty. 1 B1003:** Cross bar attached to arms in the front to maintain arch positions. **Qty. 1 LS-50M:** Motorized translation stage for focusing bottom objective. **Qty. 1 MIM-Tube-K (200):** 200 mm tube lens for bottom camera. **Qty. 1 C60-RA_Mirror:** Right angled mirror for bottom inverted microscope. **Qty. 1 C60_BeamsplitterII:** Dichroic filter cube holder for bottom inverted microscope. **Qty. 1 C60-D-Cube:** Filter cube for bottom inverted microscope.
CDZ-1000	**Qty. 1** XY precision centering stage. Used to center the diSPIM module over the bottom microscope objective. Provides 25mm X travel and 8 mm Y travel (xy resolution is 10 μm).
APZOBJ-200	**Qty. 2** Piezo-Z objective movers, 200 μm travel range. Provides closed loop control with integrated sensors.
X-Y Stage	**Qty. 1 IX-8102:** Automated X-Y sample stage. **Qty. 1 I-3078:** Sample chamber holder. **Qty. 1** DiSPIM Sample chamber.

Supplementary Table 4, Apparent size of 100 nm fluorescent beads, as viewed in each arm and after deconvolution

Bead #	SPIMA		SPIMB		After Deconvolution	
	Lateral FWHM (μm)	Axial FWHM (μm)	Lateral FWHM (μm)	Axial FWHM (μm)	Lateral FWHM (μm)	Axial FWHM (μm)
1	0.47	1.52	0.52	1.29	0.36	0.35
2	0.51	1.59	0.47	1.32	0.37	0.38
3	0.48	1.72	0.52	1.34	0.36	0.34
4	0.53	1.67	0.54	1.51	0.35	0.37
5	0.53	1.84	0.53	1.78	0.36	0.35
6	0.48	1.88	0.51	1.89	0.37	0.37
7	0.51	1.79	0.54	2.06	0.38	0.39
8	0.51	1.93	0.52	1.91	0.39	0.38
Average ± sd	**0.50 ± 0.02**	**1.75 ± 0.14**	**0.52 ± 0.02**	**1.63 ± 0.3**	**0.37±0.01**	**0.37±0.02**

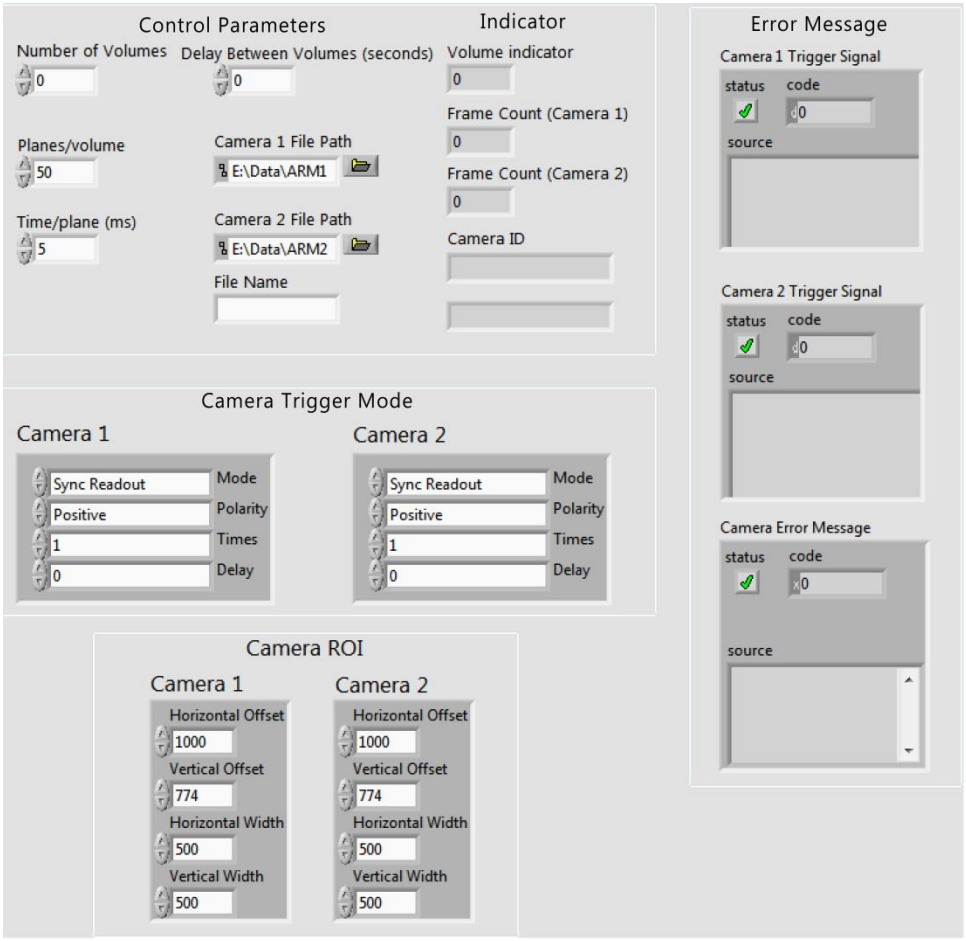
FWHM: full width at half maximum. Deconvolution was performed for 15 iterations. See also **Fig. 2**.

Supplementary Note 2, Labview vis used in diSPIM

The diSPIM data acquisition process relies on three custom vi programs, written in the Labview programming environment.

Camera Test.vi is used to test the two diSPIM cameras prior to data acquisition. Such testing is advisable in order to ensure that each camera can acquire image at 200 Hz, at durations exceeding thirteen hours. A successful test run also ensures that the cameras have been connected properly to the data acquisition computer. Camera Test.vi saves images in the Hamamatsu image format (HCIMG) to the RAID drive for fast acquisition. These files are later converted into tiff format (using Tiff conversion.vi, described further below). The front panel of this vi is shown below:

Fig. S1, Camera Test.vi front panel



A detailed description of the adjustable parameters follows:

Number of Volumes: The number of volumetric timepoints in the 4D time series collected by each diSPIM arm, i.e. entering '1' would acquire one stack for each arm.

Planes/Volume: Number of images (or planes) in each volume.

Time/Plane (ms): The combined exposure plus readout time for each plane. The minimum allowed time is 5 ms.

Delay Between Volumes (seconds): The time delay between two consecutive dual view volumes, the minimum value is currently two seconds.

Camera File Path: Provides a file path to save the data on the data acquisition computer's RAID drive. Two separate folders should be made, corresponding to each camera.

File Name: Provides a file name prefix for each image in the acquisition series. Needs to be updated every time the vi is run. Note that multiple volumes in each time series have their file names incremented automatically.

Camera Trigger Mode: The Flash 4.0 camera can run in different trigger modes. Although various trigger options can be selected (see the camera manual for more detail), we run the cameras in Sync Readout mode, because the camera acquires an image every time it receives an external trigger pulse and the hybrid global/rolling shutter exposure mode[1] can be easily realized in this mode.

Camera ROI (region of interest): The camera ROI can be changed using these offsets (see also **Supplementary Note 1 SF12**). Note that the acquisition speed depends on the camera ROI. For typical 200 Hz operation, the vertical width (Y-pixels) should be less than or equal to 500 pixels, and the vertical offset should be set according to the Y-pixel values (as described in **Supplementary Note 1 SF12**).

Commonly used parameters for testing the performance of diSPIM cameras using this vi are:

Number of Volumes: 1000

Planes/Volume: 60

Time/Plane: 5 ms

Delay Between Volumes: 2 seconds

Camera File Path: User defined

File Name: User defined

Camera Trigger Mode: Sync Readout

Polarity – Positive

Time – 1

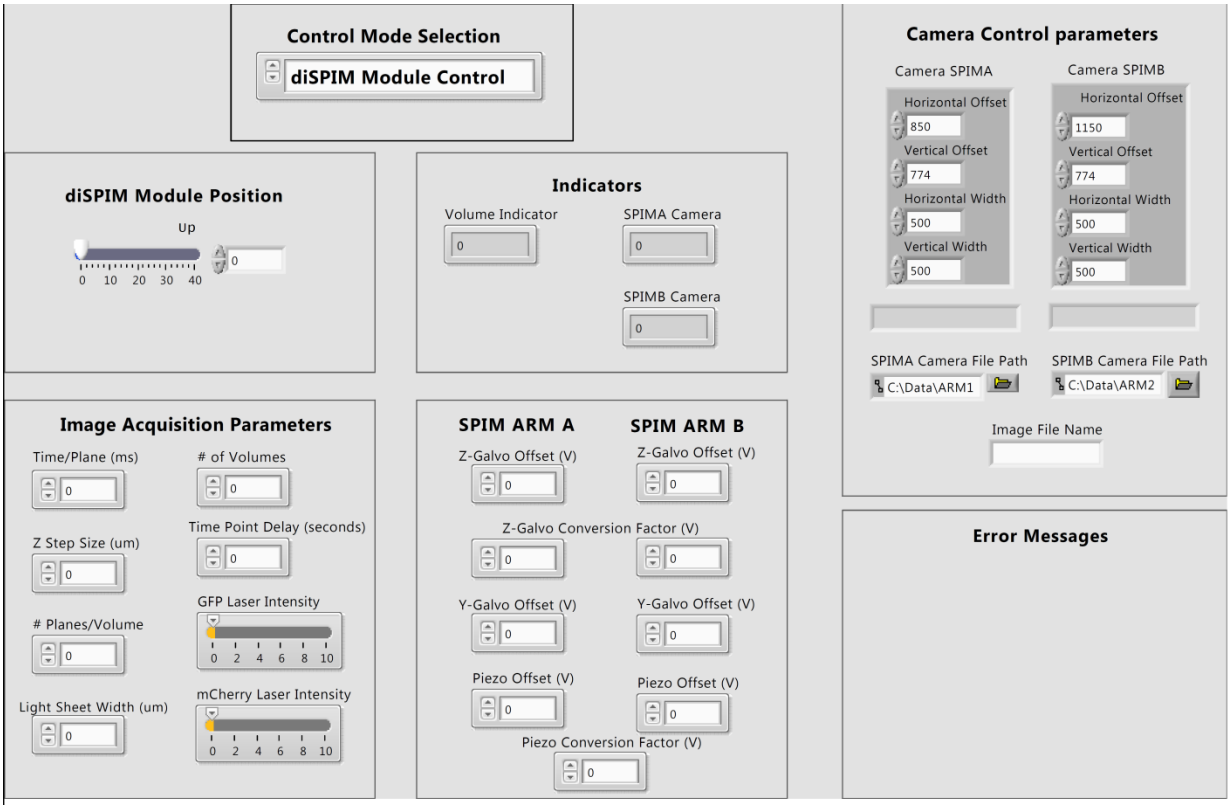
Camera ROI: Horizontal and Vertical Width: 500 pixels

Horizontal Offset: User defined (typically 100-1500 pixels)

Vertical Offset: 774

The master program that is used for controlling diSPIM alignment, characterization, testing and image acquisition is **diSPIM Control.vi**. The front panel of this vi is:

Fig. S2, diSPIM Control.vi front panel



The vi can be operated in 5 modes that share common control parameters. Only one mode can be used at a time, and mode selection is defined from the “Control Mode Selection” pull-down menu. A more detailed description of the various modes follows:

1. **diSPIM Module Control:** By selecting this mode, the diSPIM module can be moved vertically, towards and away from the coverslip surface. Entering the diSPIM module position and running the vi moves the diSPIM module to the specified position. Ensure that the diSPIM module position does not cause the objectives to touch or crack the coverslip; once a command is issued there is no way of stopping the movement. A limit on the range of the LS-50 stage can be set by appropriately positioning two magnets internal to the LS-50 (see the ASI manual for more details). Note that the position defined by this parameter is relative, not absolute; if the home or zero buttons are pressed on the joystick controller, the 'zero' position is reset. In the event that these buttons are mistakenly pressed, move the diSPIM module manually using the joystick knob, until it is within 50-100 μm of the coverslip, and set that position as 'zero'.
2. **Parameter Adjustment:** This mode is useful while aligning the system, as all diSPIM hardware except camera- and laser-specific parameters can be adjusted. This mode is often used to define the extent of the light sheet and to monitor the position of the light sheet using the bottom camera or diSPIM cameras when using manufacturer-supplied camera software. Parameters can also be changed while this vi is running. This mode does not sweep either the light sheet or piezo in the Z-direction; instead, the Z-galvo and piezo positions can be stepped by changing the relevant offset value. Offset values are defined as the central Z position during image acquisition. This mode can also be run continuously for 15 minutes (if running more than 15 minutes is desired, rerun the vi). This continuously running mode is useful in finding and placing the sample within the camera chip and within the light sheets. Also, proper overlap of the light sheets can be checked using this vi.
3. **Scouting:** This mode is used to optimize imaging parameters, by running the vi and opening and inspecting the resulting image(s) in ImageJ. Set the number of volumes to 1 and run this vi. Change the parameters, run the vi again, and inspect the effect of altered parameters on the acquired images.
4. **Image Acquisition (GFP, 488 nm):** This mode is used for image acquisition using 488 nm laser excitation. A new file name is required each time this mode is run. For time-lapse imaging, image files are incremented automatically. Raw data are saved in the Hamamatsu HCIMG format on the RAID drive and automatically converted to tiffs that are saved in a user defined folder. For each volume, one image folder containing all images is created. For time-lapse 4D imaging, folder names are also incremented. Once image acquisition is complete, the tiff images comprising each volume can be combined into a single multilayer tiff using an ImageJ macro (an example is provided in Supplementary Software).
5. **Image Acquisition (mCherry, 561 nm):** This mode is used for image acquisition using 561 nm laser excitation, e.g. for mCherry. Parameters are the same as for GFP image acquisition.

A more detailed description of the front panel parameters in **Fig. S2** follows:

diSPIM Module Position: To move the diSPIM module up/down, enter the position (in mm, relative to the coverslip) and then run the vi. When moving the diSPIM module down (towards the coverslip), we recommend setting the zero position no closer than 40 μm from the coverslip. If positioning the diSPIM module closer to the coverslip is desired, we recommend using the manual joystick knob. To move the diSPIM module away from the coverslip (e.g. for changing a sample), enter 40 and run the vi. This will move the diSPIM module up 40 mm.

Time/Plane: This determines the image acquisition time (exposure time + readout time, a minimum of 5 ms).

Z-Step Size: This determines the Z-step size (in μm) used in acquiring volumes.

# of Planes/Volume: # of planes/volume is determined by the Z-step size and total Z-range to be covered. For example, if you want to image a volume with Z extent 50 μm, at 0.5 μm Z-step size, set the # of planes/ volume 100.

# of Volumes: For time-lapse imaging, enter the number of desired volumes

Time Delay Between Volumes: For time-lapse imaging, specify the delay between successive volumes. The minimum value is 2 seconds.

GFP (488 nm) Laser Intensity: This controls the 488 nm laser intensity via the AOTF. Units are arbitrary, and the maximum value is 10 V.

mCherry (561 nm) Laser Intensity: This controls the 561 nm laser intensity.

Z-Galvo Offset: This offset value defines the central Z position of an imaging volume. The total range of Z-galvo offset is from 0-4.5 V, so we advise setting this value between 2-3.5 V to ensure that the entire imaging volume is covered when the galvo scans the light sheet in the Z direction.

Z-Galvo Conversion Factor: This is the voltage needed to move the galvo by 1 μm (typically set to 0.013 V). If scanning the light sheet in the Z direction is not desired, this value should be set to 0. To optimally synchronize the light sheet with the collection objective for volumetric imaging, small adjustments of this parameter may be needed (**Supplementary Fig. 8**). We recommend adjusting the Z-galvo conversion factor in steps of 0.001 V (we typically find the optimal value to be between 0.011 V and 0.014 V).

Y-Galvo Offset: This offset defines the light sheet position in the Y-direction (along the scan direction when creating the light sheet, or the vertical position as viewed in the bottom camera). The allowed range is 0-4.5 V, but we recommend keeping the value between 2-3.5 V. With the 40x objectives used in this protocol, the maximum light sheet width is around 90 μm.

Piezo Offset: This parameter defines the central position of both objective piezos; piezos can be moved toward (by increasing the offset voltage) or away (by reducing the offset voltage) from the coverslip by changing this value. 1 V corresponds to a 20 μm movement of the piezo. The total range for this parameter is 0-10 V, corresponding to a total movement of piezo of 200 μm.

Piezo conversion factor: This is the voltage needed to move the piezo 1 μm. For APZOBJ 200, this is 0.05 V. This parameter is normally fixed, and should be changed only if piezo motion is not desired (for example, in **Supplementary Data 6**, when determining light sheet thickness).

Camera ROI: The camera ROI can be adjusted by defining horizontal and vertical offsets as well as widths (in pixels, see also **Supplementary Note 1 SF12**).

Camera File Path: Provide a separate file path for both arms to save the acquired images. Images are saved in HCIMG format on the RAID drive, and are automatically converted into tiff format by Tiff conversion.vi (details provided below) into this user specified path. Make sure that the RAID drive data folder is cleared before starting an experiment. The default folder is (C/data/SPIMA and C/data/SPIMB). The program gives an error message if the supplied file name is already contained within the data folder.

File Name: The prefix given to each volumetric tiff file is specified here.

Error Messages: There are many potential error messages that can result when running Labview. Many error messages can effectively handled by finding the error code and looking it up in LabVIEW, or the Hamamatsu camera manual. One common mistake is to not change the file name, which results in an error because the camera is not recognized by the program.

Indicators: displays the number of images acquired using each camera, and the number of volumes for a given 4D image series.

Commonly used parameters for testing the performance of diSPIM cameras using this vi are:

Time/plane: 5 ms

Z-step size: 0.2 - 1 μm

# of Planes/Volume: Desired Z- range (in μm)/Z- step size (in μm)

# of volumes: User defined (for *C. elegans* embryo imaging*:* 1000)

Light sheet width: 60 μm

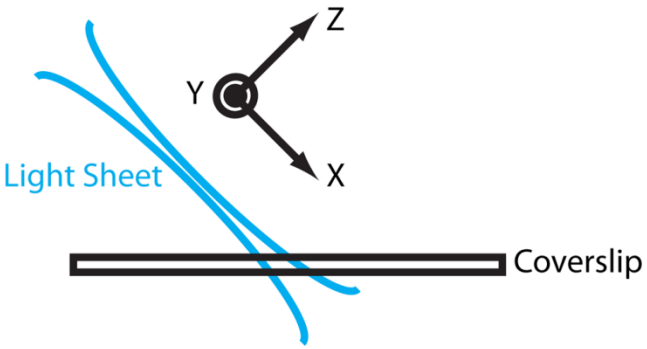
Time point Delay: 2 - 60 seconds

SPIM A and SPIM B Parameters: Same as used in scouting.

Camera ROI: For 200 Hz operation set the horizontal and vertical widths at 500 pixels and vertical offset at 774. The horizontal offset can be adjusted based on the position of the sample.

We also define the relevant axes in **Fig. S3:**

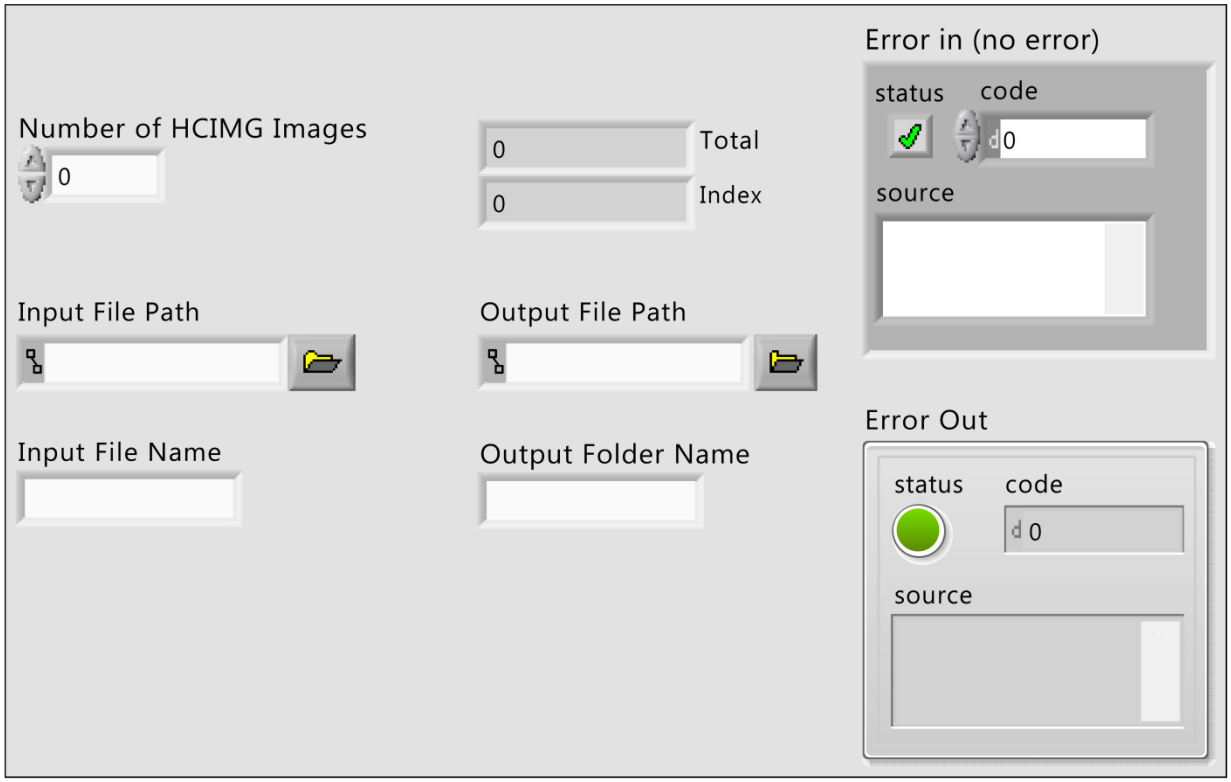
Fig. S3, Explanation of XYZ axes in galvo parameters



XYZ axes in each galvo parameter (e.g. Y-Galvo offset) are defined as above, relative to the illumination light sheet generated by that arm. The 'X' axis is defined as the light sheet propagation axis, the 'Y' axis is the direction of scan (i.e. making a light sheet from a single pencil beam), and the 'Z' axis is perpendicular to the light sheet. The 'Y' galvo creates the light sheet, and the 'Z' galvo moves the light sheet through the volume.

Finally, **Tiff conversion.vi** converts HCIMG images into tiff format. When acquiring at fast frame rates, the Flash 4.0 cameras save data in HCIMG format (one HCIMG file is saved per volume). Upon running this vi, the volumetric HCIMG file is converted to a series of tiff images (each plane is saved as a separate tiff image), and a separate folder (one per volume) is created to store these tiff images. Conversion of multiple tiff files into a multilayer tiff is accomplished by a macro (see **Supplementary Note 3**). The front panel of this vi is shown below:

Fig. S4, Tiff conversion.vi front panel



Front panel parameters are described as follows:

Number of HCIMG Images: The number of HCIMG images to be converted into tiff format.

Input and Output File Path: Provide the file path where HCIMG is saved and output file path where tiff image folder is to be saved.

Input File Name: Provide the HCIMG file name. For converting multiple files, the HCIMG image names should be incremented so that this vi can automatically convert the series of HCIMG files.

Output Folder Name: Provide an output folder name. For multiple output files, the vi automatically increments the number of the folder name.

Error Message: If the HCIMG file is not saved properly or could not be opened, an error message will be displayed.

Connecting diSPIM hardware to the DAQ cards: In order to use these Labview vis to control the diSPIM, the diSPIM hardware must be connected to appropriate analog output (AO) voltage channels on the DAQ cards. Each DAQ card has 8 analog out (AO) channels which can be accessed from the BNC extension board connected to the DAQ cards. These channels are numbered AO0 to AO8 on the BNC extension board. We suggest the following steps in order to verify the proper connections between AO channels and diSPIM hardware:

1. Open the National Instruments measurement and automation explorer (MAX) program.
2. Click on Devices and Interfaces to display the submenu.
3. Click on Dev1 to display the serial number of this DAQ card and to verify the identity of the BNC extension board connected to this DAQ. Do the same for Dev2.
4. Connect various hardware components to the BNC extension boards using the following channels:
   Dev1/ao0: SPIMA Z-Galvo
   Dev1/ao1: SPIMA Y-Galvo
   Dev1/ao2: SPIMB Z-Galvo
   Dev1/ao3: SPIMB Y-Galvo
   Dev1/ao4: SPIMB Objective
   Dev1/ao5: SPIMA Objective
   Dev2/ao0: SPIMB Camera Trigger
   Dev2/ao1: SPIMA Camera Trigger
   Dev2/ao2: 488 nm Laser control
   Dev2/ao3: 561 nm Laser control
   Dev2/ao4: AOTF blanking control
5. To test any of the above channels right click the relevant device in NI Max and open the test panel. Select the channel to be tested and select voltage sine wave generation. Connect that channel to an oscilloscope and check to see whether the sine wave is applied.

1 Wu, Y. *et al.* Spatially isotropic four-dimensional imaging with dual-view plane illumination microscopy. *Nat Biotechnol.* **31**, 1032-1038 (2013).

Supplementary Note 3, image macro for making background subtracted, cropped, multilayer tiff files from a series of individual tiff files

This macro is used to make background-subtracted, cropped, multilayered tiff images from a series of individual tiff files. The basic macro functionality will be the same each time the macro is run, but certain parts of the macro need to be changed from run to run. These portions are denoted in red and consist of file locations, size of a cropped image, and the number of image sets to run the macro on. In addition, the user should replace the specific number corresponding to the first image with the "+i+" command in order to get the macro to process all images.

```
for(i=1;i<=825;i++)

{

run("Image Sequence...",
"open=E:\\BV24\\SPIMB_Uncompiled\\bv"+i+"\\BV192_SPIMB_0000.tif number=48
starting=1 increment=1 scale=100 file=[] sort");

imageCalculator("Subtract create stack", "bv"+i+"","bg0");

selectWindow("Result of bv"+i+"");

makeRectangle(169, 102, 230, 350);

run("Crop");

saveAs("Tiff", "E:\\BV24\\SPIMB\\SPIMB_embryo"+i+".tif");

close();

run("Close");

}
```